Calmodulin Binding to Rat Adipocyte Plasma Membrane: Characterization and Photoaffinity Cross-Linking of Calmodulin to Binding Proteins†

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ABSTRACT: [^{125}I]Calmodulin binding to isolated rat adipocyte plasma membranes has been characterized, and the calmodulin binding proteins associated with the membrane have been identified by use of the photoaffinity cross-linker *N*-hydroxysuccinimidyl 4-azidobenzoate. Total binding of [^{125}I]calmodulin to plasma membranes was assayed by a centrifugation method and found to be calcium dependent, requiring 2.2 μM free calcium for half-maximal binding. Total binding was curvilinear with time, plateauing at 30 min. In addition, calmodulin binding was demonstrated to be both saturable (1700 pmol/mg of membrane protein) and exchangeable. Additional calmodulin binding sites were not produced by further ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) treatment of membranes prepared in the presence of ethylenediaminetetraacetic acid (EDTA). Eight specific calmodulin binding protein complexes were identified by use of the photocovalent cross-linking agent. Results obtained with photocovalent cross-linking were similar to those obtained in the total calmodulin binding assays. The formation of the calmodulin binding protein complexes was dependent on time and calcium concentration. The integration of these two techniques provides a powerful tool for studying calmodulin-regulated proteins.

The unique role of calmodulin resides in its ability to bind four molecules of calcium and subsequently interact with a large number of enzymes and proteins whose diverse functions are modulated by this interaction. A wide range of approaches has been devised to identify these calmodulin binding proteins. These apporaches have included the use of calmodulin affinity columns (Westcott et al., 1979), gel overlay (Gleeny & Weber, 1980), fluorescence (Pardue et al., 1981; LaPorte et al., 1981), azido-derivatized calmodulin (Andreasen et al., 1981), and bifunctional cross-linking reagents (LaPorte et al., 1979). However, some of these techniques are of limited application in their ability to characterize calmodulin's interaction with calmodulin binding proteins in membrane preparations. The development of a new technique that can be useful both in the identification of calmodulin binding proteins in membrane preparations and in the characterization of their properties would be a useful tool for experiments designed to probe the mechanism by which calmodulin interacts with its myriad of target proteins.

We report here the characterization of [^{125}I]calmodulin binding to the rat adipocyte plasma membrane. By the use of a total calmodulin binding assay we have found binding to be calcium dependent, saturable, reversible, and heterogeneous. Furthermore, we have been able to confirm these observations and, in addition, identify the specific calmodulin binding proteins by polyacrylamide gel electrophoresis of ^{125}I-labeled calmodulin, covalently bound to calmodulin binding proteins in the adipocyte plasma membrane. Integrating both techniques results in the development of a powerful tool for studying calmodulin-regulated proteins.

Materials and Methods

Materials. Collagenase (type I) from *Clostridium histolyticum*, bovine serum albumin (fraction V), and parvalbumin were obtained from Sigma Chemical Co., St. Louis, MO. *N*-Hydroxysuccinimidyl 4-azidobenzoate was purchased from Pierce, Rockford, IL. [^{125}I]Iodine was obtained from Amersham Corp., Arlington Heights, IL. All other reagents were from standard sources. S100b was the generous gift of Dr. Boyd Hartman (Washington University School of Medicine, St. Louis, MO).

Preparation of Rat Adipocyte Plasma Membranes. Male Sprague-Dawley rats were purchased from Eldridge Laboratory Animals, Barnhart, MO. Adipocytes were prepared from rat epididymal fat pads by the method of Rodbell (1964) using Krebs–Ringer phosphate buffer. The plasma membranes were prepared by a modification (Jarett, 1974) of the method of McKeel & Jarett (1970) and stored at −70 °C. Plasma membranes were routinely used within 6 weeks of preparation. Storage in this manner was found to have no effect on [^{125}I]calmodulin binding when compared to that of fresh membranes.

Preparation of [^{125}I]Calmodulin. Purified calmodulin was prepared from rat brain by the method of Charbonneau et al. (1979). The purity of the calmodulin preparation was established (migration as a single band) by NaDodSO$_4$[1]–polyacrylamide gel electrophoresis in a 12% gel and its ability to stimulate calmodulin-dependent cyclic nucleotide phosphodiesterase (Wolff & Brostrom, 1979). Biologically active [^{125}I]calmodulin was prepared by the lactoperoxidase procedure described by Graf et al. (1980). The biological activity of the iodinated vs. native calmodulin was determined by iodinating calmodulin with nonradioactive iodine and then comparing its ability to stimulate calmodulin-dependent cyclic nucleotide phosphodiesterase with native calmodulin. The activities for both native and iodinated calmodulins were found to be comparable with K_m = 9 nM calmodulin for both iodinated and native calmodulins.

[^{125}I]Calmodulin Binding to Rat Adipocyte Plasma Membranes. Binding of labeled calmodulin to rat adipocyte plasma membranes was assayed by incubating 30–50 μg of membrane

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[1] Abbreviations: NaDodSO$_4$, sodium dodecyl sulfate; HSAB, *N*-hydroxysuccinimidyl 4-azidobenzoate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

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protein in 200 μL of a buffer mixture containing 8 mg/mL bovine serum albumin, 6 mM $MgCl_2$, 0.8 mM $CaCl_2$, 100 mM NaCl, 20 mM KCl, 0.5 mM EGTA, 25 mM Tris, pH 7.4, and the appropriate amount of [^{125}I]calmodulin (0.1–0.2 μCi). Standard incubations were for 1 h at 37 °C, at which time the samples were placed on ice, and the membranes were pelleted by centrifugation at 11700*g* for 5 min with an Eppendorff microfuge. The pellets were washed twice in the above buffer and the pellets resuspended and pelleted as before to remove excess unbound labeled calmodulin. The radioactivity remaining in the pellets was determined with a γ counter. Omission of $CaCl_2$ from the buffer mixture was used to quantitate the nonspecific binding.

Photolysis and Cross-Linking of [^{125}I]Calmodulin to Rat Adipocyte Plasma Membranes. Washed membrane pellets containing bound [^{125}I]calmodulin, which were obtained from the incubation as outlined above, were next resuspended into 200 μL of ice-cold Hank's balanced salts medium, and then 4 μL of HSAB in dimethyl sulfoxide was added to give a final concentration of 50 μM. This addition was followed by a 3-min incubation after which the membranes were photolyzed for 8 min with a Blak-Ray ultraviolet lamp at an intensity of 12000 μW/cm^2 in a custom-designed, temperature-controlled irradiation cabinet. Following photolysis, the membranes were immediately pelleted, the supernatant was aspirated, and the membranes were prepared for electrophoresis by solubilization in 90 μL of a solution composed of 62 mM Tris, pH 6.7, 5% glycerol, 3% $NaDodSO_4$, and 2% β-mercaptoethanol. Prior to electrophoresis, the samples were heated at 100 °C for 3 min.

$NaDodSO_4$ Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed by the method of Laemmli (1970). Electrophoresis was performed at a constant power on a 2 mm thick 9% gel. The molecular weight standards were myosin (210000), β-galactosidase (116000), phosphorylase *a* (92500), bovine serum albumin (66000), and ovalbumin (45000). After completion of electrophoresis, the gels were stained and destained according to Fairbanks et al. (1971) with Coomassie brilliant blue R250 and dried. The dried gels were autoradiographed at −70 °C with Kodak XAR-5 film with a Du Pont Cronex Lighting-Plus intensifying screen. Quantitation of radioactivity in individual bands was determined by excising that portion of the dried acrylamide strip that corresponded to the band identified by autoradiography and counting it in a γ counter.

Results

Characterization of Total [^{125}I]Calmodulin Binding to Plasma Membranes. The interaction of calmodulin with calmodulin binding proteins present in the rat adipocyte plasma membrane was first studied by the total [^{125}I]calmodulin binding assay. Figure 1 shows the average of six experiments studying the time course of total [^{125}I]calmodulin binding to plasma membranes in the presence and absence of calcium. The binding of calmodulin with the plasma membranes in the presence of calcium occurs initially in a rapid, time-dependent manner, reaching steady-state binding after approximately 30 min of incubation. [^{125}I]Calmodulin binding in the absence of calcium is only 20% of the binding when calcium is present in the incubation mixture. This calcium-independent contribution to the total binding is considered to be nonspecific.

Labeled calmodulin bound to the plasma membrane can be exchanged with unlabeled calmodulin. The [^{125}I]calmodulin bound to the plasma membrane is displaced in a concentration-dependent manner by unlabeled calmodulin (Figure 2). The time course of displacement of [^{125}I]calmodulin by unlabeled calmodulin showed that steady-state binding had been reached by 60 min of incubation, the time of incubation used in the displacement experiments shown in Figure 2. The amount of bound [^{125}I]calmodulin remaining after incubation with 50 μg/mL unlabeled calmodulin was less than 21% of the initial bound [^{125}I]calmodulin after incubation for 5 min. The remaining exchangeable [^{125}I]calmodulin was maximally displaced after 60 min of incubation (data not shown). Additional insight into the specificity of calmodulin binding is obtained by studying the ability of structurally related Ca^{2+}-binding proteins (Kretsinger, 1980) to displace bound calmodulin from the plasma membranes. As revealed in Figure 2, S100b did show an effect, but the concentration required was 20 times greater than that for calmodulin. Parvalbumin was without effect at all concentrations tested.

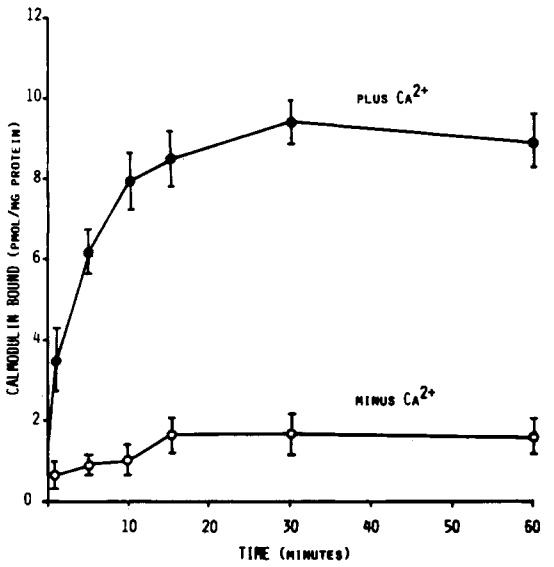

FIGURE 1: Time course of [^{125}I]calmodulin binding to rat adipocyte plasma membrane. Approximately 35 μg of plasma membrane protein prepared in the presence of EDTA was added to the ice-cold binding mixture described under Materials and Methods. The reaction was initiated by addition of [^{125}I]calmodulin to a final concentration of 0.8 μM and immediately placing the sample in a 37 °C shaker. Samples were processed and analyzed as described under Materials and Methods after the incubation period indicated. Each point represents the mean ± SEM of results obtained from six different experiments involving four different membrane preparations.

Calmodulin binding to adipocyte plasma membranes was saturatable, with saturation occurring at approximately 1700 pmol/mg of membrane protein (Figure 3). The concentration of calmodulin at which half-saturation was attained was 4 μM. Scatchard plot analysis of these results was curvilinear, which suggested either heterogeneity of calmodulin binding sites or the existence of negative cooperativity between similar sites.

Calmodulin binding to plasma membranes was dependent on the concentration of calcium added into the binding assay (Figure 4). The free calcium concentration for the half-maximal binding of [^{125}I]calmodulin was approximately 2.2 μM. A free calcium concentration of 8 μM was the standard assay condition used throughout the course of this study. In addition, we have examined the effect of various concentrations of magnesium (0–10 mM) on calmodulin binding and found that under our binding conditions, changes in magnesium concentrations produced no alterations in the calcium-dependent calmodulin binding (data not shown). These data are consistent with studies reported by other investigators (Pardue et al., 1981; Graf et al., 1980; Linden et al., 1981).

Adipocyte plasma membranes used in this study were routinely prepared in the presence of EDTA, which is though to

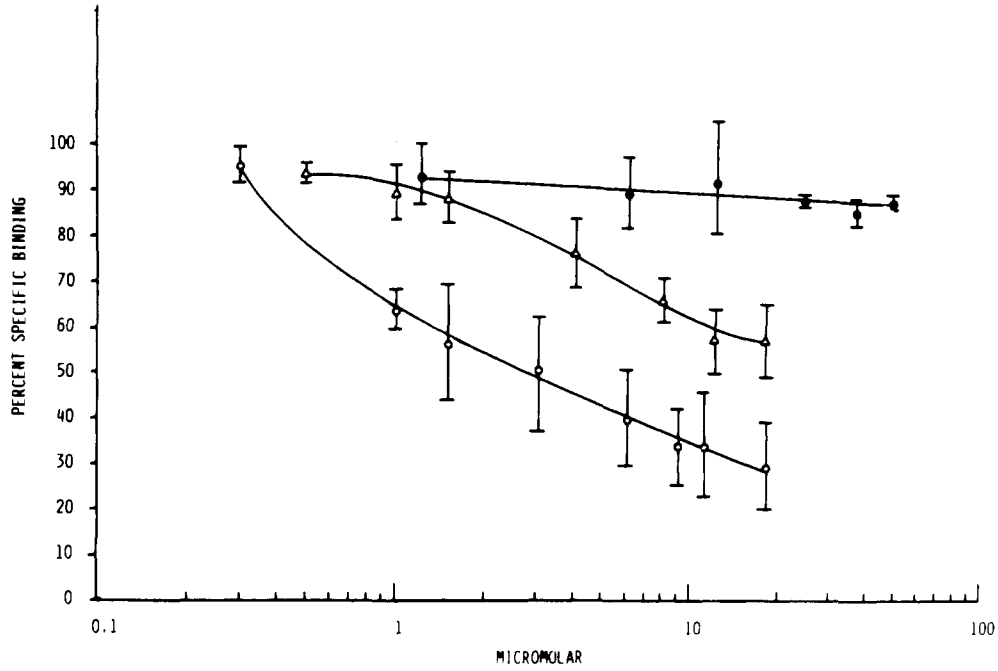

FIGURE 2: Displacement of [^{125}I]calmodulin bound to adipocyte plasma membrane by calmodulin (○), parvalbumin (●), and bovine brain S100b (△). [^{125}I]Calmodulin was bound to adipocyte plasma membranes as described under Materials and Methods. The membranes were pelleted, washed with 200 μL of buffer containing various amounts of unlabeled calmodulin, paravalbumin, or bovine brain S100b as indicated, and incubated at 37 °C for 60 min. The membranes were next pelleted by centrifugation, the supernatant was removed, and the radioactivity in the pellet was quantitated. The percent specifically bound [^{125}I]calmodulin remaining bound to the membrane after incubation at the indicated concentrations of the various competing proteins was calculated. The data presented in Figure 2 represent the mean ± SEM from three different experiments.

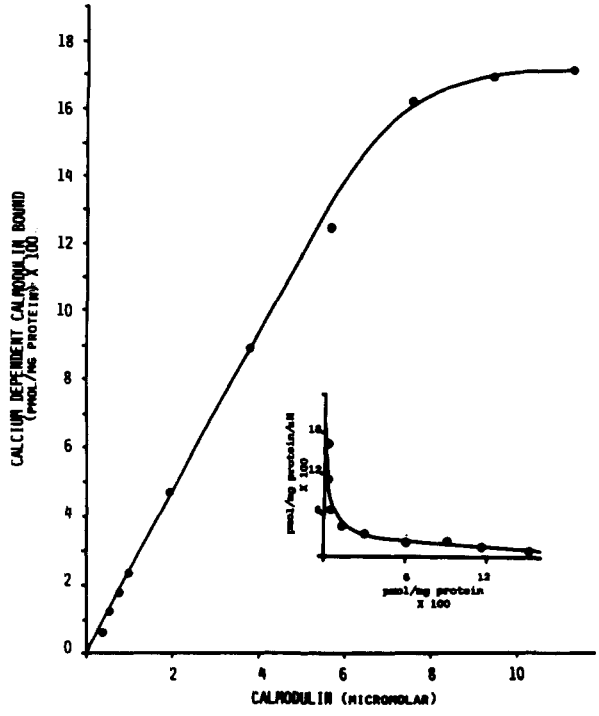

FIGURE 3: Effect of calmodulin concentration on Ca^{2+}-dependent [^{125}I]calmodulin binding to plasma membranes. Increasing amounts of [^{125}I]calmodulin were incubated for 1 h at 37 °C in 200-μL samples with 35–50 μg of adipocyte plasma membranes. Following incubation, the samples were placed on ice, and the extent of binding was determined as described under Materials and Methods. The data shown represent the average values for three separate experiments using membranes obtained from three different fractionations. The SEMs are too small to be graphically expressed. A Scatchard plot of the binding data is presented as an insert.

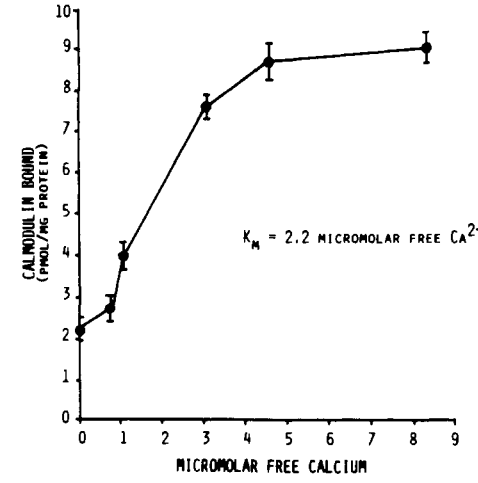

FIGURE 4: Calcium dependency of [^{125}I]calmodulin binding to adipocyte plasma membranes. Total calmodulin concentration was 1.75 μg/mL of assay, containing 50 μg of plasma membrane protein. The binding assay was made 5.0 mM in EGTA, and the total $CaCl_2$ concentration was varied to achieve the desired free [Ca^{2+}]. Free Ca^{2+} was calculated by taking into account all the species involved in the equilibrium between H^+, Mg^{2+}, Ca^{2+}, and EGTA (Pershadsingh et al., 1980). The affinity constants were for 25 °C and 0.1 ionic strength derived from the compendia of Martell & Smith (1974) except that a value of $10^{10.36}$ was used for CaEGTA.

strip the noncovalently bound calmodulin from the plasma membrane (Linden et al., 1981; Grab et al., 1979). In order to test this assumption, plasma membranes were extracted several times with buffers containing EGTA, in cycles consisting of 10-min incubations at 37 °C, and then assayed for [^{125}I]calmodulin binding. The results (Table I) demonstrated that no additional calmodulin binding sites were generated, indicating that the preparation of membranes in the presence of EDTA is sufficient to remove endogenous exchangeable calmodulin associated with the plasma membrane. Therefore, saturation-binding experiments would appear to accurately represent the maximum sites available for exchangeable

Table I: Quantitation of [^{125}I]Calmodulin Binding to Plasma Membranes following Multiple EGTA Extractions[a]

no. of EGTA extractions	[^{125}I]calmodulin bound (pmol/mg of membrane protein)		
	$+Ca^{2+}$	$-Ca^{2+}$	Ca^{2+} dependent
0	11.1	2.5	8.6
1	10.4	2.6	7.8
2	10.1	2.2	7.9
3	9.4	1.8	7.6

[a] Adipocyte plasma membranes (50 μg) were added to 200 μL of incubation mixture composed of 50 mM EGTA–Tris, pH 7.4, and incubated at 37 °C for 10 min. The membranes were next pelleted by centrifugation for 5 min at 11500*g*, and the supernatant was removed. The pellet was next either incubated with [^{125}I]calmodulin for 1 h and subsequently photo-cross-linked to its binding proteins as described under Materials and Methods or subjected to further EGTA-extraction cycles prior to binding and photolysis.

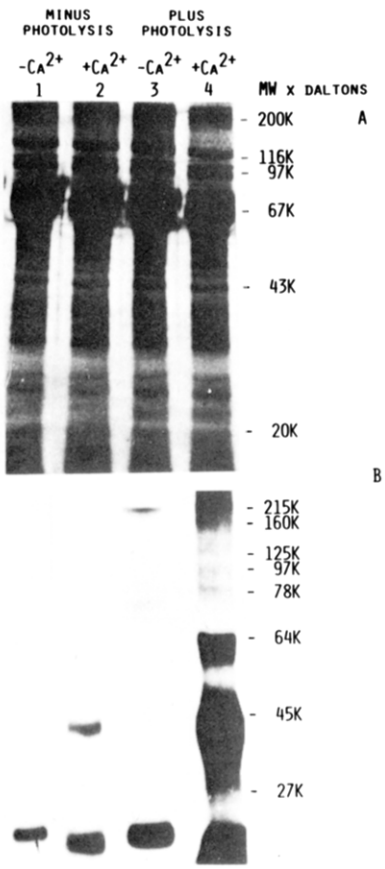

FIGURE 5: $NaDodSO_4$–polyacrylamide gel electrophoresis of rat adipocyte plasma membranes. (A) Coomassie blue staining patterns of major adipocyte plasma membrane proteins. (B) Autoradiograph showing the presence of photoaffinity cross-linked [^{125}I]calmodulin to membrane proteins. Membranes were incubated with 8×10^{-7} M [^{125}I]calmodulin at 37 °C for 1 h in the presence (lanes 2 and 4) and absence (lanes 1 and 3) of calcium. Membranes were then washed and incubated in the presence (lanes 3 and 4) or absence of 50 μM HSAB. Membranes were then irradiated (lanes 3 and 4) or incubated in the dark (lanes 1 and 2). Following irradiation the membranes were pelleted, solubilized under reducing conditions, and electrophoresed on a 9% $NaDodSO_4$–polyacrylamide gel.

calmodulin binding on the plasma membrane.

Photoaffinity Cross-Linking of [^{125}I]Calmodulin to Adipocyte Plasma Membranes. Identification and characterization of the specific calmodulin binding proteins present in the plasma membranes were conducted through the use of the bifunctional photoaffinity cross-linker HSAB. [^{125}I]Calmodulin was bound to membranes and subsequently cross-linked to its membrane binding proteins by the procedure described under Materials and Methods. The results of a typical cross-linking experiment showing both the protein (Coomassie blue) staining pattern and the corresponding autoradiograph are presented in Figure 5. At least eight bands are discernable on the autoradiograph, indicating the formation of several calmodulin binding protein complexes. The molecular weights of these bands are 215 000, 160 000, 125 000, 97 000, 78 000, 64 000, 45 000, and 27 000. Many of these bands may represent more than a single polypeptide. This is especially true of the 45 000 dalton band, which, on short exposure of the autoradiograph, is found to be composed of at least three distinct bands (data not shown). In the absence of treatment with ultraviolet light, these cross-linkage products are not observed with the exception of a 40 000 dalton band formed in the presence of calcium. Furthermore, the Coomassie blue staining patterns of standard incubations after photolysis were essentially identical with those of control incubations that were unexposed to ultraviolet light (Figure 5).

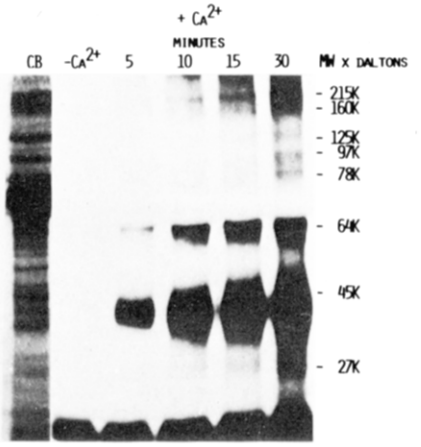

FIGURE 6: Time course of formation of photo-cross-linked [^{125}I]calmodulin binding protein complexes. [^{125}I]Calmodulin was incubated at 37 °C with adipocyte plasma membranes for 5, 10, 15, and 30 min in the presence and absence of calcium prior to photolysis. The samples were solubilized under reducing conditions and electrophoresed on a 9% $NaDodSO_4$ gel. The autoradiograph of the gel was exposed for 7 days in the presence of a Du Pont Cronex screen. Lane CB is a representative Coomassie blue stained protein lane from the gel. The lane designated $-Ca^{2+}$ is the autoradiograph of the 30-min time point incubated in the absence of calcium.

The formation of calmodulin–protein complexes in plasma membranes is calcium dependent, as demonstrated (Figure 5, lanes 3 and 4) by the minimal formation of radioactive cross-linked products in the absence of calcium. Approximately 40–50% of the total membrane-bound [^{125}I]calmodulin was dissociated from the putative membrane binding proteins during the 8-min course of photolysis as determined by quantitating the amount of [^{125}I]calmodulin bound both prephotolysis and postphotolysis. This loss may be the result of dissociation of calmodulin from low-affinity binding proteins or perhaps the result of dissociation of nonspecifically bound [^{125}I]calmodulin.

The time course for the formation of specific calmodulin binding proteins was consistent with results obtained by the total calmodulin binding assay. A typical time course of the formation of the individual calmodulin binding protein complexes in the presence and absence of calcium is presented in Figure 6. In the presence of calcium, calmodulin binding to specific proteins increased and finally plateaued. In the absence of calcium only several faint radioactive cross-linked products were observed (Figure 6). The formation of [^{125}I]-

Table II: Quantitation of [^{125}I]Calmodulin Binding Protein Complexes Resolved by NaDodSO$_4$-Polyacrylamide Gel Electrophoresis[a]

molecular weight $\times 10^{-3}$	calcium-dependent [^{125}I]calmodulin binding protein (pmol/mg of membrane protein)	% of total [^{125}I]calmodulin binding protein complexes
215	0.178 ± 0.019	4.0 ± 0.42
160	0.209 ± 0.042	5.2 ± 1.20
125	0.195 ± 0.090	4.9 ± 2.30 (variable)
97	0.222 ± 0.022	5.6 ± 0.51
78	0.221 ± 0.045	5.5 ± 1.10
64	0.506 ± 0.250	12.7 ± 7.30 (variable)
45	2.250 ± 0.988	56.5 ± 25.00 (variable)
27	0.705 ± 0.150	5.1 ± 4.00 (variable)

[a] Quantitation of incorporation of [^{125}I]calmodulin into the specific binding proteins of the plasma membrane was determined by quantitating the radioactivity present in the specific bands on the dried gel corresponding in position to the autoradiographic bands. The calcium-dependent component was calculated by subtracting the value obtained in the absence of calcium from the corresponding radioactive band obtained in the presence of calcium. The percentage of the total complexes formed for each specific [^{125}I]calmodulin binding protein was determined by dividing the specific [^{125}I]calmodulin binding protein by the sum of the total complexes. Cross-linking efficiency was computed by dividing the sum of the specific [^{125}I]calmodulin binding protein complexes by the total radioactivity present in the electrophoresis sample. The designation "variable" serves to emphasize those cross-linkage products whose formation demonstrated notable variation from preparation to preparation. Each value represents the mean ± SEM of results obtained from four different experiments involving four different membrane preparations.

calmodulin binding proteins in the absence of calcium could represent several proteins demonstrating unusually high affinities for calmodulin.

The efficiency of cross-linking between [^{125}I]calmodulin and the various membrane binding proteins was determined by quantitating the radioactivities of the gel slices that corresponded in location to the autoradiographic position of the cross-linked products. Approximately 15% of the calmodulin present in standard incubations was covalently linked to binding proteins as determined by dividing the total radioactivity found in the specific bands by the total radioactivity present in the initial incubation mixture. The quantitation of the various [^{125}I]calmodulin cross-linkage products is shown in Table II. In addition, quantitation of the individual bands is also presented and expressed in terms of the percent of the total [^{125}I]calmodulin cross-linkage products formed. Variation in this percentage was noted for several bands (Table II). These variations in stoichiometries may be due to protein lability or reflect variation in different plasma membrane preparations.

Discussion

Activation of enzymes under the control of calmodulin occurs by the binding of calmodulin to its target proteins (Cheung, 1980; Means & Dedman, 1980) in a calcium-dependent manner. This common regulatory mechanism is shared by a diverse array of enzymes. Considerable research efforts have been dedicated to identifying and elucidating calmodulin binding proteins. The methods used to identify calmodulin binding proteins have taken many forms but in general utilize the affinity properties of this protein (Westcott et al., 1979). The use of trifluoperazine and other antipsychotic drugs for the identification of calmodulin-regulated processes has gained widespread use (Gietzen et al., 1980; Wong & Cheung, 1979; Brostrom et al., 1978; Levin & Weiss, 1977, 1978, 1980; Kobayashi et al., 1979), but recent reports of the nonspecificity of these agents raise growing concern of their suitability for this purpose (Roufogalis, 1981; Osborn & Weber, 1980). In this study, we have combined the techniques of a total [^{125}I]calmodulin binding assay and a photoaffinity cross-linking method to quantitate and identify the various calmodulin binding proteins present in the plasma membrane of rat adipocyte. Studies employing only total binding assays are unable to ascertain or detect subtle changes in the interaction of calmodulin with specific binding proteins. However, the use of a photoaffinity cross-linking technique allows detection of these changes in the individual binding proteins.

Through the use of the total binding assay we have demonstrated that the total binding of [^{125}I]calmodulin to the adipocyte plasma membrane is calcium dependent, with half-maximal binding occurring at a free calcium concentration of 2.2 μM. Calcium-dependent binding of [^{125}I]calmodulin to the plasma membranes reached steady state after approximately 30 min of incubation. Interestingly, calmodulin binding to rat striatal membranes (Gnegy et al., 1980) and erythrocyte ghost membranes (Graf et al., 1980) requires a much shorter incubation to reach steady state, 2 and 15 min, respectively. However, in these experiments different concentrations of [^{125}I]calmodulin were employed (2.8×10^{-8} M and 8×10^{-8} M, respectively, compared to 8×10^{-7} M used in the present study).

Bound [^{125}I]calmodulin can be displaced by unlabeled calmodulin in a concentration-dependent manner, demonstrating the reversibility of binding of the [^{125}I]calmodulin binding protein complexes. In addition, bovine brain S100b displaces calmodulin but at concentrations 20 times greater than calmodulin, while parvalbumin has no effect at any concentration. Furthermore, [^{125}I]calmodulin saturates the plasma membrane calmodulin binding protein with a total binding capacity of approximately 1700 pmol/mg. Similar experiments using rat strial membrane report a calmodulin binding capacity of 11.8 ± 1.2 pmol/mg of protein (Gnegy et al., 1980). However, the calmodulin binding capacity of the erythrocyte plasma membrane (Graf et al., 1980) is reported to be 144 ng/mg of membrane protein. These data suggest differences in the calmodulin binding capacities of membranes from different tissues.

We have determined that preparation of plasma membranes in the presence of EDTA effectively strips all exchangeable calmodulin from the membrane. Multiple extractions of plasma membranes with EGTA produce no net increase in the amount of [^{125}I]calmodulin bound per milligram of membrane protein. Sobue et al. (1979) reported that synaptic plasma membranes contain 3.6 μg of calmodulin/mg of membrane protein when prepared in the absence of chelator. Treatment with EGTA reduced the value to 0.9 μg/mg of membrane protein. These data, taken together, indicate that the membranes prepared in the presence of chelator does strip calmodulin from the membrane, leaving a residual calmodulin component which is refractory to EGTA extraction. The effect of divalent cations on calmodulin binding was further analyzed with magnesium in a concentration range from 0 to 10 mM. No effects on either calmodulin binding or formation of cross-linkage products were observed, indicating no apparent requirement for magnesium for calcium-dependent calmodulin

binding to its membrane proteins. Andreasen et al. (1981), using bovine cerebral cortex membranes, reported similar results. In addition, they found that Mn^{2+} but not Mg^{2+} can substitute for calcium.

Through the use of the photoaffinity bifunctional cross-linking technique we have identified at least eight bands representing calcium-dependent [^{125}I]calmodulin cross-linkage products by $NaDodSO_4$ gel electrophoresis ranging in molecular weight from 27 000 to 215 000. Only minimal cross-linkage of [^{125}I]calmodulin to individual or total plasma membranes was observed when calcium was deleted from either the binding or photolysis buffer. We have identified eight major calmodulin cross-linked products in the adipocyte plasma membrane. Andreasen et al. (1981), using azido-[^{125}I]calmodulin, recently identified calmodulin binding proteins from bovine cerebral cortex membranes having apparent molecular weights of 77 000, 95 000, 107 000, 165 000, and 175 000. Several of the cross-linkage products identified in cerebral cortex membranes have approximately the same molecular weights as those found in the adipocyte plasma membrane.

One theoretical limitation of the photo-cross-linking technique is the possible formation of cross-linkage complexes that are not simple calmodulin–binding protein complexes but rather include additional polypeptide chains. This possibility may be particularly important in interpreting these types of results when examining calmodulin's interaction with dimeric molecules such as calmodulin-dependent phosphodiesterase. The results from this study cannot rule out the aforementioned limitation of this technique. However, comparison of the Coomassie blue protein bands for nonphotolyzed and photolyzed proteins suggests that this not the case (Figure 5).

Affinity cross-linking has proved a valuable approach to membrane-receptor characterization (Sahyoun et al., 1978; Pilch & Czech, 1980). Johnson et al. (1981) have recently utilized this technique to covalently link ^{125}I-labeled glucagon to a liver plasma membrane protein of M_r 53 000 and identified it as the glucagon receptor that activates adenylate cyclase. Similarly, we have found photoaffinity cross-linking of calmodulin binding proteins with radioactive calmodulin to be a valuable tool for identifying calmodulin binding proteins and studying the interaction of calmodulin with these various proteins. We have recently extended the use of these techniques to studying the direct effect of insulin on calmodulin binding to rat adipocyte plasma membranes (Goewert & McDonald, 1981).

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